Leptin Protects Host Cells from *Entamoeba histolytica* Cytotoxicity by a STAT3-Dependent Mechanism

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The adipocytokine leptin links nutritional status to immune function. Leptin signaling protects from amebiasis, but the molecular mechanism is not understood. We developed an *in vitro* model of ameba-host cell interaction to test the hypothesis that leptin prevents ameba-induced apoptosis in host epithelial cells. We demonstrated that activation of mammalian leptin signaling increased cellular resistance to amebic cytotoxicity, including caspase-3 activation. Exogenous expression of the leptin receptor conferred resistance in susceptible cells, and leptin stimulation enhanced protection. A series of leptin receptor signaling mutants showed that resistance to amebic cytotoxicity was dependent on activation of STAT3 but not the Src homology-2 domain-containing tyrosine phosphatase (SHP-2) or STAT5. A common polymorphism in the leptin receptor (Q223R) that increases susceptibility to amebiasis in humans and mice was found to increase susceptibility to amebic cytotoxicity in single cells. The Q223R polymorphism also decreased leptin-dependent STAT3 activation by 21% relative to that of the wild-type (WT) receptor (*P* = 0.035), consistent with a central role of STAT3 signaling in protection. A subset of genes uniquely regulated by STAT3 in response to leptin was identified. Most notable were the TRIB1 and suppressor of cytokine signaling 3 (SOCS3) genes, which have opposing roles in the regulation of apoptosis. Overall apoptotic genes were highly enriched in this gene set (*P* < 1E−05), supporting the hypothesis that leptin regulation of host apoptotic genes via STAT3 is responsible for protection. This is the first demonstration of a mammalian signaling pathway that restricts amebic pathogenesis and represents an important advance in our mechanistic understanding of how leptin links nutrition and susceptibility to infection.
Downstream signaling cascades activated by leptin include phosphoinositide 3-kinase (PI3K), mitogen-activated protein kinases (MAPK), mammalian target of rapamycin (mTOR), protein kinase B (AKT), and the inhibition of the AMP-dependent protein kinase (AMPK) (2, 37, 64).

The overarching antiapoptotic and prosurvival roles of leptin (8, 12, 20, 22, 28, 44, 45, 54) are of particular relevance to the pathogenesis of amebiasis, as induction of host cell apoptosis at the intestinal epithelium is required for *E. histolytica* infection (3). *E. histolytica* induces contact-dependent apoptotic alterations in host cells, including chromatin condensation, membrane blebbing, DNA laddering, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) positivity, phosphatidyserine exposure, and caspase-3 activation (6, 7, 33, 34, 52, 53). Extensive apoptotic cell death is observed in murine amebic liver abscesses, where amebic cytotoxicity can be blocked with the caspase inhibitor zVAD-FMK (3, 34). Similarly, caspase-3 knock-out mice and transgenic mice overexpressing Bcl-2 are more resistant to amebic infection (3). Contrary to canonical apoptotic pathways, however, *E. histolytica* kills cells rapidly, and loss of host cell membrane integrity is observed (5), suggesting that necrotic or pyroptotic pathways may also be involved.

Leptin is well characterized as an antiapoptotic factor for many cell types, including intestinal epithelial cells (12, 26, 45, 54, 57, 62, 63), and mice with elevated leptin levels (db/db) display increased colonic expression of caspase-3 (47). Additionally leptin concentrations are induced ~10-fold during inflammation of the colonic epithelium (11, 57). We hypothesized that leptin might play a protective role during *E. histolytica* infection by directly preventing apoptosis of host cells. In this study, leptin signaling was demonstrated to prevent amebic cytotoxicity in vitro in a STAT3-dependent manner. Expression of the long form of the leptin receptor was sufficient to confer significant resistance to amebic cytotoxicity in vitro. Further analysis of the intracellular signaling events activated by leptin revealed that STAT3 signaling is the critical regulator of resistance to amebic cytotoxicity.

**MATERIALS AND METHODS**

Leptin was purchased from A. F. Parlow, National Hormone and Peptide Program (Torrance, CA). The STAT3 inhibitor STATTIC, the PI3K inhibitor LY294002, and the MEK inhibitor PD98059 were purchased from EMD Chemicals (Gibbstown, NJ).

**Tissue culture and transfection.** HEK293T/17 cells were maintained in a humidified incubator at 37°C with 5% CO2. HEK293T/17 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM)-F-12 with nonessential amino acids, sodium pyruvate, and 10% heat-inactivated fetal bovine serum (FBS). Jurkat T cells were cultured in RPMI supplemented with 10% heat-inactivated FBS. Caco-2 cells were cultured in Eagle’s minimum essential medium (EMEM) supplemented with nonessential amino acids, sodium pyruvate, and 20% heat-inactivated FBS. Cells were discarded after passage 25, and new cultures were started from frozen stocks.

HEK293T/17 cells were transiently transfected using Lipofectamine 2000 per the product instructions (Invitrogen, Carlsbad, CA). The plasmids mLEPRb/pCDNA3 (47), mLEPRbR223/pCDNA3 (35), and LEPRb-Delta65/pCDNA3 (47) were obtained from Martin Myers and have been described previously as referenced. pCDNA3 was used as a vector control.

The mLEPRb/pCDNA3 plasmid was used as a template for site-directed mutagenesis to construct mLEPRb985LpCDNA3, mLEPRb1077L/pCDNA3, and mLEPRb1138L/pCDNA3. Site-directed mutagenesis was done using the QuikChange kit (Stratagene/Agilent, Santa Clara, CA).

The presence of the desired mutations and the absence of other mutations were confirmed by DNA sequencing.

**ImmunobLOTS.** Cell lysates were prepared in radioimmunoprecipitation assay (RIPA) buffer by incubation on ice for 30 min. Lysates were boiled for 3 min in SDS-PAGE sample buffer and immediately subjected to SDS-PAGE. Proteins were transferred to polyvinylidene difluoro (PVDF) membranes by standard wet transfer methods. Membranes were blocked in Tris-buffered saline–Tween 20 (TBST) for 1 h. Blots were probed with a primary murine antibody to the Lepr (BAF497; R&D Systems) at a 1:1,500 dilution. Secondary detection was carried out using a horseradish peroxidase (HRP)-conjugated anti-mouse antibody (Sigma-Aldrich) at 1:5,000 dilution. Bands were visualized with ECL reagents (Pierce).

**Luciferase assays.** The appropriate LEPRb and luciferase reporter plasmids were transiently transfected into HEK293T/17 cells in 96-well plates, as described above. A total of 200 ng LEPRb plasmid, 50 ng GAS-Luc (encodes STAT3-responsive gamma interferon-activated sequence (GAS) element), and 5 ng pRL-TK (encodes constitutive Renilla luciferase) were transfected per well. Luciferase activity was measured 48 h posttransfection using dual-glo luciferase reagents (Promega, Madison, WI) for quantification of both Renilla and firefly luciferase. Firefly luciferase values were normalized to Renilla luciferase values to control for transfection efficiency and cell number.

**Cytotoxicity assays.** (i) **LDH release.** A total of 5 × 10^5 cells/well were aliquoted into a microtiter plate in 100 μl of M199 (Gibco) supplemented with cysteine, bovine serum albumin (BSA), and HEPES (M199S). *E. histolytica* strain HM1:IMSS trophozoites were washed three times in M199S and resuspended to concentrations to yield the indicated infection ratios. Trophozoite suspensions (100 μl) were added to each well. Plates were incubated 1 to 3 h at 37°C with 5% CO2. At the end of the incubation period, plates were centrifuged at 500 × g for 5 min, and 50 μl of supernatant from each well was transferred to an opaque microtiter plate. Lactate dehydrogenase (LDH) levels in the supernatant were measured using the CytoTox-ONE homogeneous membrane integrity assay (Promega, Madison, WI) as directed. Briefly, 50 μl reconstituted cytox-1 reagent was added to each well. Plates were incubated for 10 min at 22°C. A total of 25 μl of stop solution was added to each well, and fluorescence was quantified at 560 excitation/590 emission using a Spectramax M2 plate reader (Molecular Devices, Sunnyvale, CA). Percent cytotoxicity was calculated relative to a maximum LDH release condition, and 100% cell lysis was accomplished by the addition of 0.2% Triton-X per well. All conditions were tested in triplicate. Each experiment was repeated at least 3 times, and representative experiments are shown.

(ii) **Caspase-3 activation.** A total of 5 × 10^5 HEK Jurkat, or Caco2 cells/well as appropriate were pipetted into a microtiter plate in 50 μl OptiMEM (Gibco). *E. histolytica* trophozoites were washed three times in phosphate-buffered saline (PBS) and then dounce homogenized in homogenization buffer (10 mm Tris [pH 7.4], 0.25 M sucrose) followed by centrifugation at 500 × g for 5 min. A total of 50 μl of lysate was added to each well, and the plates were incubated for 1 h. Caspase activation was measured using the Apo-ONE homogeneous caspase-3/7 assay (Promega, Madison, WI). Briefly, caspase-3 reagent was reconstituted, and 100 μl of caspase reagent was added to each well of the microtiter plate and mixed by pipetting. Plates were incubated for at least 3 h at room temperature in the dark before fluorescence was read at 485 excitation/527 emission using a Spectramax M2 plate reader (Molecular Devices, Sunnyvale, CA). Amebic lysate did not activate the caspase-3 substrate in the absence of host cells. Cells were also treated with homogenization buffer alone to ensure that caspase-3 activation was due to the addition of amebic lysate. Additionally, controls were performed to ensure that amebic lysate alone does not cleave the Ape-ONE substrate (data not shown). All conditions were tested in triplicate. Each experiment was repeated at least 3 times, and representative experiments are shown.

**qRT-PCR.** HEK293 cells were transfected with wild-type (WT) leptin receptor, Q223R LepR, Y1138L (STAT3 mutant) LepR, or an empty vec-
tor control (pcDNA). Forty-eight hours posttransfection, cells were made quiescent by incubation in optimum plus 0.5% BSA for 3 h. Cells were then stimulated with 100 ng of leptin or maintained in optimum plus 0.5% BSA for an additional 3 h. RNA was prepared using the RNeasy minikit (Qiagen). The concentration of RNA was measured using a NanoDrop 1000 spectrophotometer (Thermo Scientific) before reverse transcription using superscript II reverse transcriptase ( Invitrogen). Quantitative real-time PCR (qRT-PCR) was performed using the iCycler (Bio-Rad) and 2× GoTaq qPCR Master Mix (Promega) with a total reaction volume of 50 μl. Threshold cycle ($C_T$) values for triplicate runs were normalized to expression levels of an endogenous control, glycerinaldehyde dehydrogenase (GapDH).

Microarray analysis. HEK293 cells were transfected with WT LepR or Y1138L (STAT3 mutant) leptin receptor. Forty-eight hours posttransfection, cells were made quiescent by incubation in optimum plus 0.5% BSA for 3 h. Cells were then stimulated with 100 ng of leptin or maintained in optimum plus 0.5% BSA for an additional 3 h. RNA was prepared using the RNeasy minikit (Qiagen), and its quality was verified in an Agilent bioanalyzer. Total RNA was amplified and converted to cDNA using the 3‘ IVT Express kit ( Affymetrix). Three biological replicates of each sample were hybridized to human genome U133 Plus 2.0 gene chips (Affymetrix) and read using the GeneChip Scanner 3000 7G (Affymetrix).

RESULTS

Leptin treatment decreased amebic cytotoxicity in cells that endogenously express the leptin receptor. To determine if leptin signaling contributes to cellular response to *E. histolytica in vitro*, intestinal epithelial Caco2 cells and Jurkat T cells, both of which express a functional LepR (21, 57), were treated with leptin prior to challenge with *E. histolytica* trophozoites. Leptin treatment was protective in vitro at 5 to 500 ng/ml, as evidenced by a dose-dependent decrease in both caspase-3 activation (Fig. 1A) and cell lysis (Fig. 2C) relative to levels in vector-transfected controls after a 3-h preincubation period with 100 ng/ml of leptin to stimulate leptin receptor signaling (*P < 0.005*). Expression of LepR in the absence of leptin stimulation also caused a modest increase in resistance to cytotoxicity, possibly due to low levels of basal activation of the receptor (Fig. 2B and C). Expression of Δ65 LepR, an intracellular truncation of LepR that abrogates all downstream leptin signaling except JAK2 (34), did not confer protection from amebic killing, similarly to vector-transfected cells. Expression of Δ65 LepR in the absence of leptin stimulation did not increase resistance to cytotoxicity, indicating that no basal activation of the receptor occurs without the signaling domain (Fig. 2B and C). Additionally, leptin stimulation had no significant effect on cells expressing Δ65 LepR (Fig. 2B and C). Finally, HEK293T cells expressing WT LepR strongly induced STAT3-dependent transcription in response to leptin as measured by a STAT3-driven luciferase reporter, whereas Δ65 LepR did not induce STAT3 in response to leptin (Fig. 2D). These data demonstrate that protection from amebic cytotoxicity requires activation of intracellular signaling via LepR and that STAT3 was activated by leptin only in cells expressing LepR.

Protection from *E. histolytica* cytotoxicity in vitro was dependent on leptin signaling via STAT3. Leptin binding by the long form of LepR results in autophosphorylation of constitutively bound JAK2 (2, 14). JAK2 then phosphorylates Tyr985, Tyr1077, and Tyr1138 in the cytoplasmic domain of LepR, which act as docking sites for downstream signaling molecules. Phosphorylation of Tyr985 creates a binding site for the tyrosine phosphatase...
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Cytotoxicity was measured by vector-transfected (pcDNA) cells. (B) The effect of LepR expression on amebic cytotoxicity was measured by *E. histolytica*-mediated caspase-3 activation. HEK293T cells transfected with wild-type LepR (WT LepR), an intracellular truncation of LepR that abrogates the majority of downstream leptin signaling (Δ65 LepR), or an empty-vector control (pcDNA) were treated with 0 or 100 ng/ml leptin for 3 h prior to amebic challenge. *E. histolytica* was added at a ratio of 1 parasite to 5 host cells. Caspase-3 activation was measured after 1 h. (C) Host cell lysis by *E. histolytica* was measured by LDH release assay concurrently with caspase-3 activation. Leptin treatment significantly increased protection from cytotoxicity in cells expressing WT LepR (P = 0.04 for caspase-3 activation and cell lysis) but not Δ65 LepR or pcDNA. Additionally, the WT LepR conferred significant protection relative to both Δ65 LepR and pcDNA in the presence and absence of leptin for both caspase-3 activation (B) and cell lysis (C) (*, P < 0.05; **, P < 0.005). (D) STAT3 induction was measured in HEK293T cells transfected with WT LepR and Δ65 LepR by cotransfection with a STAT3-driven luciferase reporter. Cells were treated with 0 to 1,000 ng/ml of leptin for 3 h, and STAT3 induction in response to leptin was measured by luciferase reporter activity. WT LepR significantly induced STAT3 in response to leptin relative to Δ65 LepR and pcDNA (*, P < 0.05).

SHP-2, SOCS3 is a negative regulator of leptin signaling and also binds Tyr985. STAT5 and STAT3 bind to phospho-Tyr1077 and phospho-Tyr1138 respectively, and are activated by JAK2 phosphorylation. To determine which signaling pathways were critical for leptin-mediated protection from amebic lysis (Fig. 3B) (P < 0.05 and 0.01, respectively). These data indicate that functional recruitment and activation of STAT3 is necessary for leptin-mediated protection from amebic cytotoxicity.

STAT3 inhibition reversed the protective effect of leptin in intestinal epithelial cells. To further test the role of STAT3 activation as the critical mediator of leptin protection, cells were treated with the small-molecule STATTIC, a highly specific inhibitor of STAT3 (56). A dose response assay showed that STAT3 activation by leptin in HEK293T cells expressing LepR was nearly completely inhibited by STATTIC at the recommended concentration of 40 μM (Fig. 4A). The addition of STATTIC reversed the protective effect of LepR expression on amebic caspase-3 activation in HEK293T cells in the presence and absence of leptin but had no effect on cells lacking LepR (transfected with the vector alone) (Fig. 4B). As expected, the pan-caspase inhibitor ZVAD-FMK decreased caspase-3 activation in HEK293T cells regardless of LepR expression or leptin treatment (Fig. 4B). The difference in scale for caspase-3 activation seen in Fig. 4B is due to using 10-fold more cells for this assay; the assay remains linear over this range of cell concentrations. STATTIC inhibited STAT3 activation by lysed by *E. histolytica*.
naling, the PI3K inhibitor LY294002 and the MEK inhibitor PD98059 were tested in HEK293T cells expressing the leptin receptor. Neither inhibitor had an effect on caspase-3 activation by *E. histolytica* in HEK293 cells expressing LepR and thus were not tested further (data not shown). These data reiterate that the main leptin signaling pathway mediating protection from amebic cytotoxicity acts via STAT3. Taken together, these data show that inhibition of STAT3 reverses the protective effect of leptin on amebic activation of host cell apoptosis.

The Q223R LepR polymorphism decreased leptin-mediated protection. The Q223R polymorphism in LepR was first identified as significantly increasing susceptibility to amebiasis in humans, and this observation was later validated in the murine model of amebiasis (17, 27). To investigate whether this polymorphism's effect on susceptibility to amebiasis was also mediated by STAT3, we expressed the WT and Q223R LepR in HEK293T cells. We then compared leptin-dependent STAT3 activation and amebic cytotoxicity assays in both cell lines. Cells expressing Q223R LepR were more susceptible to amebic caspase-3 activation (Fig. 5A) and cell lysis (Fig. 5B) than were cells expressing the WT leptin receptor. This effect was significant for caspase-3 activation (*P* = 0.04) but not for cell lysis (*P* = 0.07). While the difference in cytotoxicity between the WT and Q223R LepR was greater in the absence of leptin, it was also notable upon leptin stimulation but did not reach statistical significance, possibly because the Q223R mutation diminishes receptor autoactivation in the absence of ligand. Similarly, in STAT3-transcriptional activation studies, the WT LepR displays higher basal activation of STAT3 than the Q223R LepR mutant in the absence of leptin (0.13 WT versus 0.06 Q223R; *P* = 0.035). The polymorphic Q223R LepR also displays approximately 20% less STAT3-dependent transcription than the WT LepR in response to leptin stimulation (*P* < 0.05) (Fig. 5C). Taken together, these data imply that the mechanism of increased susceptibility of the Q223R LepR may be diminished basal signaling via LepR.

**Differential gene expression in cells expressing WT versus Q223R LepR.** Several known STAT3-regulated genes with defined proliferative and antiapoptotic functions were tested for expression differences in cells expressing the wild-type or a Q223R or STAT3 mutant (Y1138L) LepR (Fig. 6). Gene products tested included STAT3, suppressor of cytokine signaling 3 (SOCS3), Survivin (BIRC5), and Bcl-xL. Induction of STAT3 expression was not induced strongly by leptin via the leptin receptor. Neither inhibitor had an effect on caspase-3 activation by *E. histolytica* in HEK293 cells expressing LepR and thus were not tested further (data not shown). These data reiterate that the main leptin signaling pathway mediating protection from amebic cytotoxicity acts via STAT3. Taken together, these data show that inhibition of STAT3 reverses the protective effect of leptin on amebic activation of host cell apoptosis.

**Identification of leptin-activated STAT3-dependent transcripts.** To identify genes involved in STAT3-dependent resis-
tance to amebic cytotoxicity, we compared the transcriptional response to leptin of the resistant wild-type LepR and the susceptible STAT3 mutant LepR (Y1138L), using the human 133 Plus 2.0 expression GeneChip microarray from Affymetrix containing 54,000 probe sets for 20,000 human genes. Imposing a false-discovery rate (FDR) cutoff of 5%, 45 genes were differentially expressed in cells expressing a wild-type LepR versus those cells with STAT3 mutant LepR (see Fig. S1A in the supplemental material), independent of exogenous leptin treatment. Similarly, 55 genes were differentially expressed in response to leptin stimulation, independent of LepR STAT3 binding status (see Fig. S1B in the supplemental material). Sixty-nine genes exhibited a significant STAT3 mutant LepR effect that differed with leptin treatment and/or exhibited a significant leptin response that differed by LepR STAT3 binding status (Fig. 7). Of these, only SOCS3 and TRIB1 exhibited statistically significant (FDR of <5%) interaction effects between leptin stimulation and mutational status. SOCS3 was strongly induced by leptin via the wild-type LepR but not by the STAT3 mutant LepR. Likewise, 10 other genes (including PNPLA8, which is regulated in response to feeding and fasting [66]; DDX3, which regulates apoptosis [55]; and NFKBIZ, which may negatively regulate STAT3 [67]) showed similar SOCS3-like patterns of dysregulation but did not achieve significance at the 5% FDR cutoff (Fig. 7). SOCS3 is a critical transcriptional repressor with well-defined roles in protecting cells from various forms of apoptosis (25) and is regulated by STAT3. PRPF6 (with marginal FDR of <10%) is involved in pre-mRNA splicing, acting as a bridging factor between U5 and U4/U6 snRNPs in formation of the spliceosome (71). The transcript most highly induced by leptin via the STAT3 mutant LepR, while not induced by wild-type LepR in response to leptin, was TRIB1, a pseudokinase important for regulating mitogen-activated protein kinase kinase (MAPKK), CCAAT/enhancer-binding protein (C/EBP), activating transcription factor 4 (ATF4), and C/EBP-homologous protein (CHOP) (68). The role of TRIB1 in regulating C/EBP, ATF4, and CHOP is intriguing in this context, as these genes are thought to activate endoplasmic reticulum (ER) stress-induced apoptosis (23). Other genes that exhibited similar TRIB1-like expression patterns, while not reaching significance at the 5% FDR cutoff, include the proapoptotic LF10, BCL10, EMP1, ID1/2, STK17B, PAMIP1, and MCL1 genes (Fig. 7) (16, 25, 50, 61, 65, 70).

Genes involved in proliferation and/or apoptosis were the major functional category identified as dysregulated via microarray analysis (27 of 69, with hypergeometric $P$ values of association less than $1 \times 10^{-5}$). The wild-type LepR strongly induced the antiapoptotic SOCS3 gene (9), while the STAT3 mutant LepR displayed increased transcription of proapoptotic genes. These results suggest that downregulation of proapoptotic genes via SOCS3 and other transcriptional repressors may be the main mechanism of increased resistance to amebic cytotoxicity regulated via LepR.

**DISCUSSION**
In this study, we explored the functional significance of leptin signaling and a common, clinically relevant polymorphism in the leptin receptor on host susceptibility to amebiasis. A reductive...
approach of testing the direct role of leptin signaling during *E. histolytica*-host cell interactions recapitulated human and mouse studies demonstrating the protective effect of leptin during amebic infection.

Using an *in vitro* model of amebiasis, we demonstrated that stimulation of leptin signaling increased resistance to amebic cytotoxicity and that this protection required expression of LepR (Fig. 1). Based on these results, we found that expression of LepR in HEK293T cells was sufficient to protect these cells from amebic cytotoxicity and that leptin stimulation increased resistance only in cells expressing LepR (Fig. 2). Together, these results clearly demonstrated that leptin signaling, rather than the presence of LepR at the cell surface, was critical for the resistant phenotype.

To further explore the downstream signaling pathways activated by leptin that could be responsible for the resistant phenotype, we constructed a series of mutant receptors, each deficient for activation of a particular leptin-signaling mediator. Using these constructs, we demonstrated that activation of SHP-2 and STAT5 via LepR was dispensable for leptin-mediated protection. However, activation of STAT3 was required for protection from amebic cytotoxicity (Fig. 3). Interestingly, cells expressing the Y1138L STAT3 mutant LepR were even more susceptible to amebic cytotoxicity than vector-transfected cells, although this effect was not significant (Fig. 3).

We also demonstrated that the specific small molecule inhibitor of STAT3, STATTIC, reversed the protective effect of leptin in transfected HEK293T cells on amebic caspase-3 activation, comparably to mutation of the STAT3 activation domain of LepR shown in Fig. 3. STATTIC had no effect on amebic cytotoxicity for HEK293T cells expressing only the empty vector, nor did it increase amebic cytotoxicity in Caco2 cells in the absence of leptin. Indeed, inhibition of HEK293T cell STAT3 signaling with STATTIC counteracted the protective effect of leptin, but STAT3 inhibition did not increase cellular susceptibility in unstimulated cells. This result is consistent with observations in HEK293T cells and indicates that STAT3 must be induced (by leptin, in this case) in order to observe a negative effect of STAT3. This makes sense, as very little active STAT3 transcription is seen in the absence of leptin stimulation in both HEK293T and Caco2 cells (Fig. 4A and D).

These results are limited by the *in vitro* nature of our experiments, as physiologic activators of STAT3 other than leptin may not be present in tissue culture medium. Indeed, *in vivo* studies of amebiasis demonstrate that both SHP-2 and STAT3 are required for leptin-mediated protection (27), hinting at a more complex protective signaling mechanism during infection of the intestinal epithelium. STAT3 is well recognized for activating production of antimicrobial molecules and inflammatory mediators, as well as promoting growth and regeneration at the intestinal epithelium (18, 32).

We used the *in vitro* system to test the effect of the common and clinically relevant Q223R LepR variant. Cells expressing the ancestral 223Q LepR were more resistant to *E. histolytica*-mediated caspase-3 activation than cells expressing the 223R leptin receptor. Interestingly this effect was more pronounced in the absence of leptin stimulation (Fig. 5). The Q223R LepR activated significantly less STAT3 than the WT LepR in response to leptin (Fig. 5) suggesting that this is the likely mechanism of increased susceptibility. The Q223R polymorphism did not significantly alter gene expression levels of four LepR-regulated genes by quantitative PCR (Fig. 6), and a recent study has shown that this polymorphism has no effect on adiposity (60). The consequences of the Q223R polymorphism on LepR function are not understood; therefore, it is not known if the
decrease in STAT3 activation is due to reduced binding affinity for leptin, reduced surface expression, or some other effect of Q223R. If the Q223R substitution does reduce LepR affinity for leptin, it is possible that high concentrations of leptin would obscure subtle differences in receptor activation. Indeed, trace levels of leptin in the medium may be sufficient to activate the WT LepR but not the Q223R LepR, and a higher-affinity interaction with leptin would account for this observation. It is also possible that the 223R LepR does not dimerize as efficiently as WT LepR and that this reduces basal activation in the absence of ligand. These possibilities warrant further investigation, as previous studies have shown that the domain of LepR containing the Q223R polymorphism is dispensable for leptin binding (35, 36, 48). Thus, while we have shown that the biological impact of the Q223R polymorphism on amebiasis is likely via reduced levels of STAT3 transcription, the mechanism by which this occurs remains unknown.

As STAT3 was required for resistance to amebic cytotoxicity and is an essential transcriptional activator, we used expression arrays to define the STAT3-regulated transcriptional response to leptin. Comparison of the global cellular transcriptional response to leptin in cells expressing WT LepR versus STAT3 mutant LepR identified SOCS3 and TRIB1 as particularly important for the STAT3-dependent leptin resistance. SOCS3 is highly induced by WT LepR in response to leptin but not by STAT3 mutant LepR, while the converse is true for TRIB1, which is highly induced by leptin via the STAT3 mutant LepR but not by WT LepR. Consistent with its role as a repressor, the main mechanism of action of SOCS3 in mediating resistance to amebiasis may be via suppression of cell death genes, as evidenced by the increased expression of proapoptotic genes in cells transfected with the STAT3 mutant LepR. Interestingly, SOCS3 acts mainly at the posttranslational level by blocking JAK-STAT interactions and targeting binding partners for ubiquitination and proteasome degradation (15, 69); thus, these changes would not necessarily be evident by gene expression array. SOCS3 gene expression is clearly diminished in the absence of STAT3, and STAT3 is reduced by the Q223R LepR polymorphism, but this does not appear to account for differences in susceptibility observed between the Q223R and R223 leptin receptors, as both induced similar levels of SOCS3 gene transcription in response to leptin (Fig. 6).

TRIB1 is highly induced in the absence of STAT3 signaling by LepR. TRIB1 is a pseudokinase thought to interact with various signaling pathways. TRIB1 may increase cellular susceptibility to E. histolytica via its interactions with MAPKK, C/EBP, ATF4, and CHOP, all of which play a role in regulation of apoptosis. Interestingly, TRIB1 and SOCS3 expression were also inversely regulated in a study of the JAK2(V617F) polymorphism in essential thrombocytosis (51). These data, combined with our observations, indicate that SOCS3 may be a repressor of TRIB1. The SOCS3 and TRIB1 genes represent interesting and biologically plausible downstream mediators of leptin-activated resistance to amebic cytotoxicity with related yet opposing roles. The specific roles of both genes during the host-E. histolytica interaction are under investigation.

Also of note are the large expression differences between STAT3 mutant and WT LepR-expressing cells regardless of leptin treatment: PERP, IFI6, CASP8, and TBX3 were highly expressed in cells expressing the STAT3 mutant LepR but not the WT LepR, while FOXP2, DDAH2, and SERINC3 were low in cells transfected with STAT3 mutant LepR but highly expressed in cells expressing a WT LepR (see Fig. S1 in the supplemental material). The differences in the transcriptional backgrounds of cells expressing WT and STAT3 mutant LepR indicates that signaling is active via the transfected receptor even in the absence of leptin stimulation. These differences clearly affect resistance to amebic cytotoxicity, as cells expressing the WT LepR were protected relative to vector-transfected cells or cells expressing the STAT3 mutant LepR even in the absence of leptin stimulation (Fig. 3).

Together, these data demonstrate the importance of leptin signaling during host-pathogen interaction. Leptin has long been implicated in regulation of host susceptibility to infection, but the mechanisms have been incompletely understood. Here, we identified STAT3 as the direct signaling pathway responsible for increased cellular resistance to E. histolytica activated by leptin. Additionally, we confirmed observations from mouse and human studies that the Q223R LepR polymorphism is detrimental to host response to amebiasis at a cellular level. Lastly, our results implicated SOCS3 and TRIB1 as potential direct effectors of host resistance to amebic cytotoxicity. This work identified the signaling cascade initiated by leptin as directly activating cellular defense to amebic cytotoxicity. The mechanism by which this occurs remains unknown.

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